Effect of geographic isolation on genetic differentiation in *Dendroctonus pseudotsugae* (Coleoptera: Curculionidae)

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Genetic structure of phytophagous insects has been widely studied, however, relative influence of the effect of geographic isolation, the host plant or both has been subject of considerable debate. Several studies carried out on bark beetles in the genus *Dendroctonus* evaluated these factors; nonetheless, recent evidence has shown that genetic structuring is a more complex process. Our goal was to examine the effect of geographic isolation on genetic structure of the Douglas-fir beetle *Dendroctonus pseudotsugae*. We used mtDNA cytochrome oxidase I (COI) sequences and RAPD markers. One hundred-seventy-two individuals were obtained from 17 populations, for which we analyzed 60 haplotypes (among 172 sequences of COI gene, 550 bp long) and 232 RAPD markers (7 primers). Analyses of molecular variance (AMOVA and SAMOVA), F-statistics and linear regressions suggest that the genetic structure of *D. pseudotsugae* is strongly influenced by geographic distance. We found that *D. pseudotsugae* has high intra- and inter-population genetic variation compared with several other bark beetles. Genetic differences among populations based on COI and RAPD markers were correlated with geographic distance. The observed genetic differences between northern (Canada–USA) and southern (Mexico) populations on *Pseudotsuga menziesii* var. *glauca* confirm that these two sets of populations correspond to previously assigned subspecies.

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The genetic structure of phytophagous insects has been widely studied (Roderick 1996), however, the factors by which their populations become genetically differentiated have been subject of considerable debate (Peterson and Denno 1998; Van Zandt and Mopper 1998). Principal among these often-intertwined factors are effects of geographic isolation and host plant influences. Several studies carried out on bark beetles in the genus *Dendroctonus* Erickson (Coleoptera: Curculionidae: Scolytinae) evaluated the effect geographic isolation (Namkoong et al. 1979; Stock et al. 1984; Roberds et al. 1987; Six et al. 1999; Zúñiga et al. 2006; Schrey et al. 2008) and of host plants (Stock and Amman 1980; Sturgeon and Mitton 1986; Langor and Spence 1991; Amman and Stock 1995; Kelley et al. 2000) on the genetic differentiation of their populations. While these studies carried out at both fine and coarse geographic scales documented genetic differences among populations associated with these factors, there is no agreement on the roles geographic isolation or host use have played in genetic differentiation in *Dendroctonus* species. In addition, recent phylogeographic evidence has shown that genetic structuring of these beetles is a more complex process (Maroja et al. 2007; Mock et al. 2007; Anducho-Reyes et al. 2008).

The distribution of genetic variability appears to be affected by the population dynamics of these insects (including eruptive and non-eruptive periods, dispersal potential), the geographical distribution both of insects and their hosts, as well as geomorphologic histories that determine different levels of allopatry or sympatry among populations (Kelley et al. 1999; Maroja et al. 2007; Mock et al. 2007; Anducho-Reyes et al. 2008; Schrey et al. 2008).

Our goal was to examine the effect of geographic isolation on genetic structure of *Dendroctonus pseudotsugae* (Douglas-fir beetle). This bark beetle colonizes one single host, *Pseudotsuga menziesii* (Mirbel) Franco across its geographic range, which has two varieties: a costal variety (var. *menziesii*) found in the Pacific northwest, and an interior variety (var. *glauca*) found throughout Rocky Mountains and north of Mexico (Hermann and Lavender 1990). Perhaps the most intuitive hypothesis of the effect of geographic isolation on genetic structure is the model of isolation by distance (IBD) (Wright 1943), which predicts that genetic differentiation between populations increases with geographic distance. Peterson

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and DENNO (1998) found by meta-analysis that IBD in phytophagous is less common in highly mobile insects (dispersal >20 km) compared with those whose vagility is low or moderate. Nevertheless, it has been shown that a fundamental factor that promotes IBD among populations is the equilibrium between gene flow and drift (HUTCHINSON and TEMPLETON 1999).

Focusing on Douglas-fir beetle found on *P. menziesii* var. *glauca* (and thus, reducing the effect of host plant), we assess the distribution of genetic variation and isolation by distance (IBD) in a north–south direction among populations. Considering the original geographical distribution of this host variety in North America, we expect both strong genetic structuring and IBD among North American beetle populations and those from Mexico. To address these questions, we examine genetic variation in mitochondrial (mtDNA) cytochrome oxidase I (COI) and random nuclear markers (RAPD) in *D. pseudotsugae* samples from across its distribution range.

MATERIAL AND METHODS

Samples and DNA extraction

A total of 172 adult *Dendroctonus pseudotsugae* were collected from 17 geographically distinct populations of Douglas-fir *Pseudotsuga menziesii* var. *glauca* from Canada, USA and México (Fig. 1A, Table 1). For comparative purposes, one population was collected from the other variety, *P. menziessi,* var. *menziesii* (Mount Hebo, Oregon). Specimens from northern populations were collected by directly sampling under the bark of recently infested trees or using traps baited with attractant pheromones, whereas almost all individuals from southern populations were collected from recently infested trees. To avoid analysis of genetically related individuals from those populations collected manually, we gathered individuals from six or seven trees per population. All beetles were stored in 100% ethanol.

Mitochondrial COI amplification

PCR amplification of a 600 bp fragment of mtDNA cytochrome oxidase I (COI) was carried out using primers C1-J-2441 (5′ACA GGW ATT AAA ATT TTT AGT TGA TTA GC 3′) and T12-N-3014 (5′TTC AAT GCA CTA ATC TGC CAT ATT A 3′) (SIMON et al. 1994). DNA amplification was performed using a Biometra T Gradient thermocycler (Biometra GmbH, Göttingen, Germany). Each PCR reaction mixture contained 50 ng of DNA, 1 µl primer 50 pM, 4 µl dNTP's 10 mM (1 µl for each dNTP), (dNTPs, Invitrogen™, Sao Paulo, Brazil), 6 µl $MgCl_2$ 25 mM, 5 µl 1× buffer, and 0.4 µl Taq DNA polymerase (Taq DNA polymerase, Recombinant, Invitrogen™, Sao Paulo, Brazil). The final volume was brought to 25 µl with ultrapure water. Cycling parameters were: preheating of samples for 10 min at 95°C, followed by 35 amplification cycles of 5 min at 94°C, 1 min at 51°C, 2 min at 72°C, and a final extension of 5 min at 72°C. Amplification products were separated on 1.5% w/v agarose gel, and stained with ethidium bromide. PCR products were purified using GFX™ PCR DNA and gel band purification kit (Amersham Biosciences, Buckinghamshire, UK) to remove primers and unincorporated dNTPs prior to sequencing. Cycle sequencing reactions were performed with BigDye fluorescent chemistry reaction (Applied Biosystems, Foster City, CA). Both forward and reverse strands were sequenced with an ABI 377 sequencer and contiguous sequences constructed and edited manually using Sequence Navigator ver. 1.0.1 (Applied Biosystems, Foster City, CA). Multiple alignments of sequences were assembled with Clustal X ver. 1.83 (THOMPSON et al. 1997). Reference sequences of each haplotype were deposited in the GenBank nucleotide sequences database (accession no. EU043405-EU043464; http://www.ncbi.nlm.nih.gov/Genbank).

RAPD-PCR amplification

Random amplified polymorphic DNA (RAPD) analysis of nuclear genome samples using a set of single short oligonucleotide primers results in a rapid production of large amounts of genetic information (WILLIAMS et al. 1990). RAPD-PCR is a versatile method that can generate useful nuclear markers for genetic structure analysis (PARKER et al. 1998; HOY 2003). Criticized for its lack of reproducibility and contamination issues (PÉREZ et al. 1998), we address these issues as described below.

The following primers were selected for RAPD-PCR analysis based on their polymorphism, brightness and consistent banding pattern: OPAM-07, OPAM-11, OPAM-13, OPB-10, OPB-01, OPM-01 and OPT-05 (Eurofins MWG Operon, Huntsville, AL). Amplification, including cycling parameters, followed the methods described for COI, with the exception that each PCR reaction contained 20 ng of genomic DNA. RAPD amplification products were separated on 2% w/v agarose gel in TAE buffer (Tris-HCL 10 mM, glacial acetic acid, 1 mM EDTA pH 8.0) at 120 V for 90 min. Gels were stained as described above and photographed with an Alpha Imager™ 2200 (Alpha Innotech Corporation, San Leandro, CA).

The reproducibility of our RAPD analyses was tested in five individuals randomly selected from the complete sample pool. Amplifications were performed twice for each primer in two independent assays, and

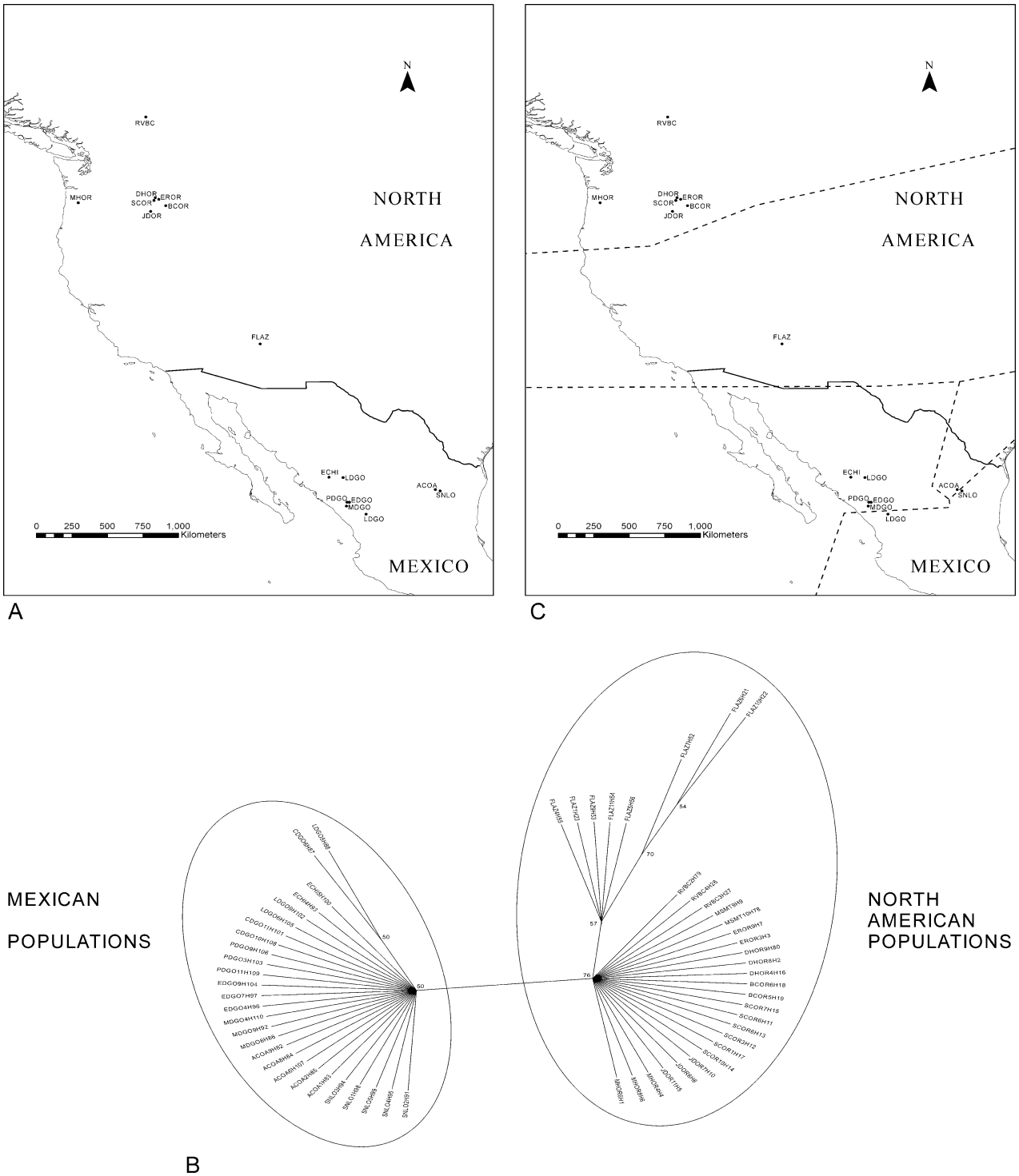

Fig. 1A–C. Sample localities of *Dendroctonus pseudotsugae* and results from SAMOVA and maximum likelihood (ML) analyses. (**A**) The geographic range of Douglas fir beetle corresponds to that of its host, *Pseudotsuga menziesii*. (**B**) Groups found in SAMOVA analysis are shown within dashed lines. (**C**) Phylogenetic analysis by ML using only the mitochondrial data set of *Dendroctonus pseudotsugae*. The best fit model of nucleotide substitution was TrN+G+I. Bootstrap values at nodes (500 pseudoreplicates).

all tests were conducted in the same laboratory using the same enzyme, reagent brand and thermocycler (Pérez et al. 1998). To determine if the total DNA of bark beetles also contain DNA from microorganisms (bacteria and yeast) associated with them, we performed PCR on bark beetle DNA using specific

Table 1. *Locations, geographic references, and number of Dendroctonus pseudotsugae specimens analyzed. Population specific haplotypes are shown in bold, along with haplotype population frequencies.*

Pop. no.	Localities	Pop. key	Latitude	Longitude	No. individuals	Haplotypes
	Southern populations					
1	Ejido Cienega de la Vaca, San Dimas, DGO.	CDGO	24° 05′ 20″ N	105° 31′ 00″ W	9	**H87**, **H101**, **H108**, H110(6)
2	Ejido Puentesillas, San Dimas, DGO.	PDGO	24° 21′ 10″ N	105° 54′ 39″ W	11	**H103**, H104, **H106**, H109, H110(7)
3	Ejido La Manga, San Dimas, DGO.	MDGO	24° 22′ 08″ N	105° 58′ 15″ W	9	H86(2), **H92(2)**, H94(2), H98(2), H110
4	Ejido Nuñez, San Dimas, DGO.	EDGO	24° 22′ 29″ N	105° 55′ 39″ W	10	**H96(3)**, **H97**, H98(2), H104(2), H110(2)
5	Santa Rita, Nuevo. León	SNLO	25° 09′ 12″ N	100° 08′ 41″ W	7	**H91**, H94 (2,), **H95**, H98(2), **H99**
6	Arteaga, Coahuila.	ACOA	25° 26′ 14″ N	100° 42′ 30″ W	12	**H81**, **H82(2)**, **H83**, **H84(3)**, **H85**, **H107(4)**
7	Llano Grande, Guanaseví, DGO.	LDGO	26° 04′ 16″ N	106° 17′ 15″ W	9	H88(4), H98, **H102(2)**, **H105**, H109
8	Ejido El Nopal, Guadalupe y Calvo, CHI.	ECHI	26° 05′ 31″ N	107° 02′ 11″ W	9	H86, H88, **H93(2)**, **H100**, H110(4)
	Northern populations					
9	Snow Bowl, Flagstaff, AZ.	FLAZ	35° 17′ 54″ N	111° 42′ 54″ W	13	**H21(2)**, **H22**, **H23(3)**, **H52**, **H53**, **H54**, **H55**, **H56(3)**
10	John Day, OR.	JDOR	44° 34′ 53″ N	118° 31′ 21″ W	11	H4(2), **H5**, H6(2), **H8**, H9(2), H10(3)
11	Balm Creek Reservoir, OR.	BCOR	44° 58′ 49″ N	117° 33′ 44″ W	11	H4(4), H6(5), **H18**, **H19**
12	Mount Hebo, OR.	MHOR	45° 10′ 30″ N	123° 40′ 08″ W	11	**H1**, H4(2), H6(6), H27, H28
13	Spring Creek, OR.	SCOR	45° 20′ 25″ N	118° 18′ 50″ W	11	H4(2), H10, **H11**, **H12**, **H13**, **H14**, **H15**, **H17**, H79(2)
14	Mt. Emily Road, OR.	EROR	45° 25′ 29″ N	118° 08′ 32″ W	9	**H3(3)**, H4(4), H7(2)
15	Drum Hill, OR.	DHOR	45° 27′ 53″ N	118° 11′ 25″ W	10	**H2**, H6(7), **H16**, **H80**
16	Lubrecht Experimental Forest, Missoula, MT.	MSMT	46° 53′ 10″ N	113° 26′ 55″ W	11	H4, H6(2), H7(2), **H9**, H27, H28(2), H78, H79
17	Revelstoke, British Columbia.	RVBC	51° 08′ 15″ N	118° 16′ 26″ W	9	H4, H6, H27(3), H28(2), H78, H79

primers for the bacterial 16S gene (Delalibera et al. 2005; Vasanthakumar et al. 2006), and yeast 26S gene (Fell 1993; Zúñiga unpubl.). All PCR tests focused on identifying DNA from bacteria and yeasts were negative, which indicates that the extraction kit and specific method used for obtaining total DNA decreased the possibility of non-beetle DNA contamination.

RAPD-PCR amplification products were scored visually; fragment size was calculated using a 100 bp DNA Ladder (Invitrogen™), manually coded, and analyzed in two different ways: 1) as phenotypic characters where RAPD bands of each locus were registered for analysis as binary data of presence (1) or absence (0), and 2) as allelic variants having a dominant inheritance pattern. Allele frequencies of RAPD bands were estimated according to Zhivotovsky (1999); we only selected those markers whose frequencies in all samples were between 0.05% and 98% (Lynch and Milligan 1994).

Statistical analyses

To assess the patterns of molecular diversity of mtDNA sequences, we estimated the haplotype and nucleotide diversity (Nei 1978) using the software DNASP ver. 4.50.3 (Rozas et al. 2003). Genetic diversity of RAPD markers was calculated using three methods: Nei's genetic diversity (h) using allelic frequencies (Nei 1973); expected heterozygosity per population (H) and average heterozygosity (H_w), using allele frequencies according to Zhivotovsky's Bayesian method (Zhivotovsky 1999); and by Shannon's index (S), calculated assuming that each phenotypic marker represents a distinct locus (Allnutt et al. 1999).

To define groups of *D. pseudotsugae* populations maximally differentiated from each other, we used both a priori (AMOVA) and a posteriori (SAMOVA) approaches. For AMOVA analysis, we performed phylogenetic analyses by maximum likelihood (ML) method using only the mtDNA data set. We used MODELTEST 3.7 (Posada and Crandall 2001) to select the best fit model of sequence evolution. The ML analysis was performed with heuristic search and TBR as swapping algorithm. The method was validated through 500 pseudoreplicates by bootstrap analysis. Additionally, although RAPD data have been considered unreliable in phylogenetic reconstruction (Backeljau et al. 1995; Hoy 2003), we chose to concatenate mitochondrial and RAPD data from each individual. This matrix was used to construct a dendrogram by neighbour-joining (NJ) with the p-distance, which allows performing a simple comparison of number of differences among individuals using both data sets. The dendrogram was validated through a bootstrap analysis with 1000 pseudoreplicates. No sister taxa were used as outgroups, because our phylogenetic reconstructions were not performed for the purpose of revealing phylogenetic relationships per se. All reconstructions were carried out using the program PAUP* (Swofford 2002).

Based on recovered groups by ML and NJ analyses, we performed AMOVA analysis using Arlequin ver. 3.0 for mtDNA data and Winamova ver. 1.55 for RAPD data (Excoffier et al. 1992, 2005) to partition the molecular variance into different hierarchical levels: within sampling sites (populations), among populations within groups, and between groups. The statistical significance of partitioned molecular variance and the associated estimate of φ-statistics were assessed by conducting 10 000 random permutations of the data (Excoffier et al. 1992).

The spatial analysis of molecular variance using SAMOVA 1.0 (Dupanloup et al. 2002) was performed using the mitochondrial data set alone. We carried out this analysis despite the fact that sampling points of *D. pseudotsugae* are not geographically adjacent and populations are not genetically homogeneous in the region under study, as it is assumed by this approach. The method implemented in this programme indirectly detects genetic barriers and defines groups of populations geographically homogeneous and maximally differentiated from each other. We ran this program repeatedly, changing n (number of groups) from 2 to 16. Significance tests were performed with 1000 permutations.

To determine whether the genetic structure of *D. pseudotsugae* describes an IBD pattern, several approaches were followed. First, linear regression of least squares between $F_{ST}/1–F_{ST}$ (using φ_{ST}) and geographic distances among the populations was conducted (Rousset 1997). Ordinary linear regressions for mtDNA COI and RAPD were independently carried out with ln-transformed geographic distances. The Mantel test was used to determine statistical significance (Mantel 1967) after 5000 random permutations using NTSYSpc ver. 2.02j (Rohlf 1998). In addition, the pairwise p-distances of the concatenated matrix (mtDNA COI sequences plus the RAPD bands of each individual) and geographical distances were used to plot a linear regression of least squares, and the non-parametric Mantel test (as described above) was also used to determine statistical significance.

RESULTS

Genetic diversity

From the 172 mtDNA cytochrome oxidase I (COI) sequences, 60 different haplotypes of 550 bp length were identified. The populations with the highest number of haplotypes were Spring Creek (SCOR), Missoula (MSMT) and Flagstaff (FLAZ) with nine, eight and eight haplotypes, respectively. The most frequent haplotypes were H4, H6 and H110, which were found in most populations. The remaining haplotypes had a frequency lower than 5%, however, most of them were population-specific (Table 1). Mean haplotype and nucleotide diversities were $\bar{h} = 0.945 \pm 0.009$ and $\pi = 0.027 \pm 0.001$, respectively (Table 3). Estimates of diversity ($\bar{h}$ and π) were not statistically different among populations, or between northern and southern populations ($P > 0.05$). Also, there was no correlation between latitude and haplotype diversity per population or between latitude and nucleotide diversity per population (data not shown).

A total of 232 RAPD markers were obtained from 172 beetles analyzed with seven primers. Size of RAPD bands selected for analysis ranged between 300–2050 bp, and reproducibility tests showed that amplifications were highly consistent and reproducible among randomly selected beetles (Table 2). Nei's genetic diversity, heterozygosity per population (H_j), and Shannon's index are shown in Table 3. Mean Nei's genetic diversity was 0.26 (SE = 0.002); the lowest value was found in the Flagstaff (FLAZ) population (0.178, SE = 0.002), while the highest occurred in the La Manga (MDGO) population (0.319, SE = 0.002). Mean heterozygosity was 0.323 (SE = 0.001); the lowest heterozygosity occurred in the Flagstaff (FLAZ) population (0.197, SE = 0.002) and the highest in the La Manga (MDGO) population (0.372, SE = 0.002). Mean Shannon's index was high (I = 0.389, SE = 0.001); the lowest (0.199, SE = 0.0004) and highest (0.482, SE = 0.0004) values were found in the same two populations (FLAZ and MDGO, respectively). As in the case of $\bar{h}$ and π from mtDNA COI data, the genetic diversity (S and h) showed no difference between southern (Mexico) and northern populations (USA and Canada) ($P > 0.05$). No correlation between latitude and genetic diversity (S and h) per population, or between latitude and expected heterozygosity per population, were found (data not shown).

Genetic structure

The ML topology of the 172 individuals using only mtDNA sequences showed two clearly distinguishable groups: one composed only by northern populations and the other only by Mexican populations (Fig. 1B). The combined data set of 782 characters (COI 550 bp + 232 RAPD markers) for 172 individuals analyzed by NJ (using p-distance) gave a dendrogram similar to the ML analysis, and recovered the same two groups of populations (Appendix 1).

The results of the AMOVA analysis using both mtDNA COI and RAPD data (φ-statistics, sum of squares, variation percentage, and probability (P) associated with φ values) are summarized in Table 4. The source of variation between groups of populations (φ_{CT}) was assessed using the two groups found by phylogenetic analysis. Both types of markers were consistent in describing the partitioning of genetic variation according to the source of variation, and revealed that variation within populations is much greater than between populations or between groups. In particular, the φ_{ST} (assessed either with mtDNA or RAPD data) showed strong genetic differentiation, and all φ-statistics were statistically different from zero, indicating that genetic variation was geographically structured.

The results obtained by SAMOVA analysis are shown in Fig. 1C and Table 5. This approach detected five groups of populations geographically differentiated ($F_{CT} = 0.631$), separated from each other by inferred genetic barriers (Fig. 1C). Three groups contained only populations from Mexico, while the two remaining groups contained only populations

Table 2. *Primers, fragment size, number of polymorphic bands and autosimilarity values with RAPD-PCR markers in D. pseudotsugae populations.*

Primer	Sequence	Fragments (bp)	Polymorphic bands	S_{XX}
OPAM-07	5′-AACCGCGGCA-3′	300-2050	35	0.785
OPAM-11	5′-AGATGCGCGG-3′	300-2050	35	0.868
OPAM-13	5′-ACCGGCACAA-3′	300-2050	32	0.805
OPB-10	5′-CTGCTGGGAC-3′	300-2050	33	0.724
OPB-01	5′-GTTTCGCTCC-3′	300-2050	29	0.701
OPM-01	5′-GTTGGTGGCT-3′	300-2050	35	0.732
OPT-05	5′-GGGTTTGGCA-3’	300-2050	33	0.863

Table 3. *Haplotype and nucleotide diversities estimated for COI data, and Nei's genetic diversity (h), heterozygosity (H_j) and Shannon index estimated for RAPD data in D. pseudotsugae populations. SE = standard error.*

Pop.	Haplotype diversity (SE)	Nucleotide diversity (SE)	Nei's genetic diversity (SE)	H_j(SE)	Shannon index (SE)
southern populations					
CDGO	0.583 (0.0023)	0.003 (0.00017)	0.295 (0.0022)	0.355 (0.00052)	0.444 (0.0012)
PDGO	0.618 (0.0022)	0.003 (0.00017))	0.267 (0.0023)	0.343 (0.00054)	0.403 (0.0014)
MDGO	0.889 (0.0014)	0.022 (0.0003)	0.319 (0.0021)	0.372 (0.0017)	0.482 (0.0013)
EDGO	0.867 (0.0014)	0.015 (0.00024)	0.255 (0.0024)	0.329 (0.00054)	0.382 (0.0013)
SNLO	0.905 (0.0017)	0.013 (0.00024)	0.198 (0.0023)	0.255 (0.00054)	0.225 (0.0014)
ACOA	0.848 (0.0015)	0.018 (0.00024)	0.305 (0.0022)	0.358 (0.00054)	0.461 (0.0014)
LDGO	0.806 (0.0019)	0.016 (0.00034)	0.303 (0.0022)	0.362 (0.00057)	0.455 (0.0015)
ECHI	0.806 (0.0019)	0.015 (0.0003)	0.271 (0.0023)	0.332 (0.00057)	0.412 (0.0014)
northern populations					
FLAZ	0.910 (0.0013)	0.027 (0.00024)	0.178 (0.002)	0.197 (0.0017)	0.199 (0.00038)
JDOR	0.800 (0.0015)	0.007 (0.00017)	0.246 (0.0023)	0.308 (0.00052)	0.377 (0.0011)
BCOR	0.709 (0.0017)	0.008 (0.00024)	0.285 (0.0023)	0.341 (0.0017)	0.431 (0.0014)
MHOR	0.705 (0.002)	0.007 (0.00024)	0.254 (0.0023)	0.326 (0.00052)	0.386 (0.0012)
SCOR	0.964 (0.0012)	0.011 (0.00017)	0.268 (0.0023)	0.328 (0.00057)	0.408 (0.0015)
EROR	0.722 (0.0017)	0.002 (0.00017)	0.295 (0.0022)	0.349 (0.00052)	0.448 (0.0016)
DHOR	0.533 (0.0023)	0.005 (0.00024)	0.235 (0.0024)	0.307 (0.00052)	0.357 (0.0013)
MSMT	0.945 (0.0013)	0.012 (0.0003)	0.229 (0.0024)	0.305 (0.00057)	0.345 (0.0013)
RVBC	0.889 (0.0016)	0.014 (0.00024)	0.263 (0.0023)	0.332 (0.0014)	0.399 (0.0014)
Mean	0.945 (0.00052)	0.027 (0.00017)	0.26 (0.0023)	0.323 (0.00084)	0.389 (0.0013)

from the North America. Mexican groups were composed as follows: group 1, ARTG from Coahuila; group 2, STAR, LMNG and ENNZ from southern Durango and Nuevo León; and group 3, PNTS, CNVC, LNGR and ENPL from northern Durango and Chihuahua. The two groups from the North America were: group 4, FLGS from Arizona and group 5, MTHB, JHDR, SCLG, BCRK, DRHL, EMRD, MSSL and CBRT from northwestern USA and southwestern Canada. SAMOVA results showed a clear differentiation due to a genetic barrier between the Mexico and the North America populations. This analysis also suggests that within these two regions genetic barriers may exist between populations of Sierra Madre Oriental and Sierra Madre Occidental, as well as between those from northwestern and southwestern USA.

Isolation by distance

Regression analyses of $F_{ST}/1–F_{ST}$ versus ln-transformed geographic distances with COI mtDNA and RAPD markers revealed no positive and monotonic relationship between genetic differentiation and geographic distance within southern populations (r Mantel = 0.161, P = 0.216, COI data; r Mantel = 0.244, P = 0.194, RAPD data), nor within northern populations (r Mantel = –0.324, P = 0.116, COI data; r Mantel = 0.591, P = 0.06, RAPD data) (Fig. 2A–B). However, when all populations sampled in this study were considered, they described an isolation by

Table 4. *Analysis of molecular variance (AMOVA) of RAPD and COI data from D. pseudotsugae populations, including DF, sum of squares, percentage of variation explained, P-values and Φ statistics. Upper values, RAPD data. Lower values, COI data.*

Source of variation	DF	SS	Percentage of variation (%)	P	Φ Statistic
Between groups (Φ_{CT}) (north and south)	1	98.458	2.01	<0.001	0.020
	1	4.292	7.72	0.01	0.077
Between populations (Φ_{SC})	15	900.892	19.6	<0.001	0.200
	15	15.181	12.11	0.01	0.131
Within populations (Φ_{ST})	155	2706.932	78.38	<0.001	0.216
	155	62.102	80.16	0.01	0.198

Table 5. *Fixation indices corresponding to the groups of populations inferred by SAMOVA for the D. pseudotsugae populations tested for the mtDNA sequences. *P <0.01; **P <0.001*

No. of groups	F_{CT}	F_{ST}	F_{SC}
2	0.595**	0.705**	0.272**
3	0.615**	0.689**	0.194**
4	0.624**	0.677**	0.139**
5	0.631**	0.659**	0.076**
6	0.630**	0.656**	0.071**
7	0.621**	0.643**	0.059**
8	0.618**	0.636**	0.047**
9	0.618**	0.635**	0.045**
10	0.614**	0.624**	0.027**
11	0.614**	0.613**	−0.001**
12	0.616**	0.612**	−0.011**
13	0.619**	0.603**	−0.041**
14	0.617**	0.595**	−0.057*
15	0.619**	0.593**	−0.069*
16	0.622*	0.591**	−0.083*

distance pattern (r Mantel = 0.231, P = 0.005, COI data; r Mantel = 0.258, P = 0.001, RAPD data) (Fig. 2C).

The linear regression of pairwise p-distances (mtDNA COI + RAPD data) and geographic distances also show that the simple number of differences among 172 individuals was positively related with their geographic distance (r Mantel = 0.488, P = 0.0004, Fig. 3). In most cases, pairs of geographically close individuals are less differentiated than those separated by longer distances. Therefore, while the effect of geographical isolation on genetic differentiation is insignificant for relatively geographically close populations, northern and southern groups of populations differ significantly.

DISCUSSION

Genetic diversity

mtDNA haplotype and nucleotide diversities have been used as estimators of intraspecific genetic diversity of many insects groups. The nucleotide diversity estimate for mtDNA COI in *D. pseudotsugae* ($\pi = 0.027 \pm 0.001$) is higher than in *D. mexicanus* Hopkins (0.011 ± 0.009, Anducho-Reyes et al. 2008), *D. rufipennis* Kirby (0.018 ± 0.004, Maroja et al. 2007) and *D. ponderosae* Hopkins (0.006 ± 0.003, Mock et al. 2007), but lower than in *D. valens* LeConte (0.028 ± 0.022, Cognato et al. 2005a) and other Scolytinae (Langor and Sperling 1997; Stauffer et al. 1997; Kohlmayr et al. 2002; Cognato et al. 2003). Our data also reveal that

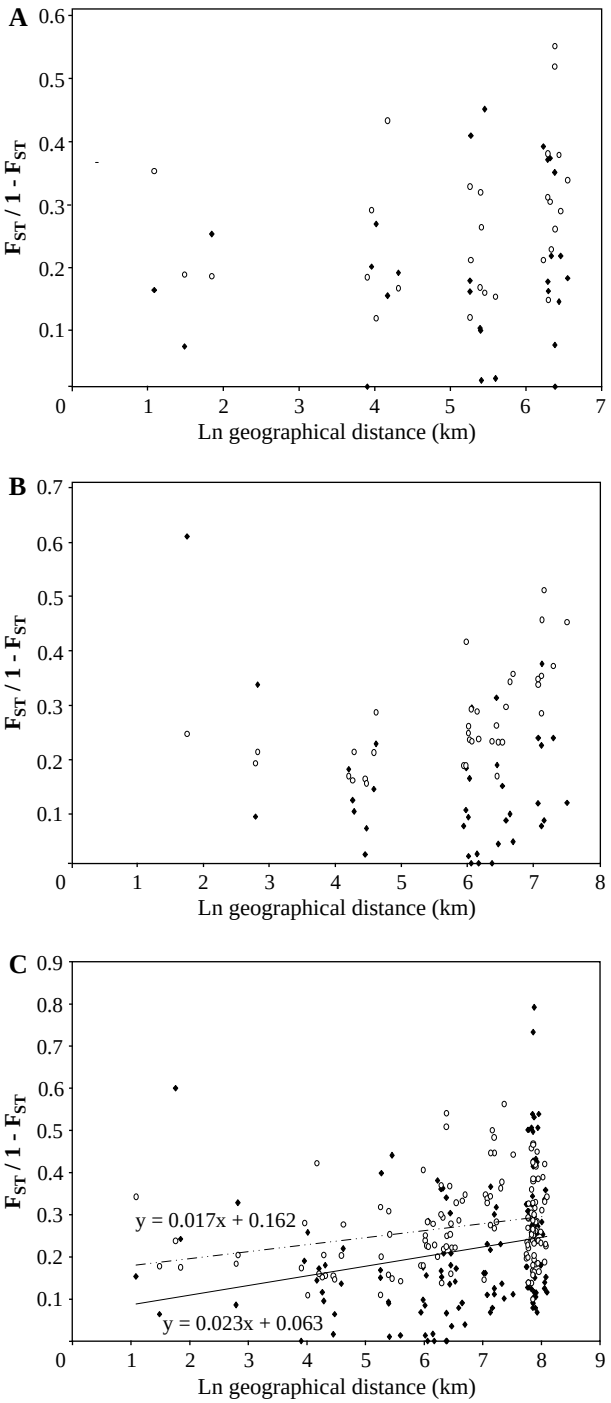

Fig. 2. Scatter plot showing the relationship between genetic dissimilarities (estimated as $F_{ST}/1 - F_{ST}$) and logarithms of geographical distances of *D. pseudotsugae* populations. (**A**) southern populations. (**B**) northern populations. (**C**) all 17 populations. Diamonds and continuous line slope, COI data; open circles and dotted line slope, RAPD data. Regression equations reported only for significant relationships.

D. pseudotsugae is a highly polymorphic species ($\bar{h} = 0.945 \pm 0.009$), as shown by the high number of different haplotypes and their frequencies.

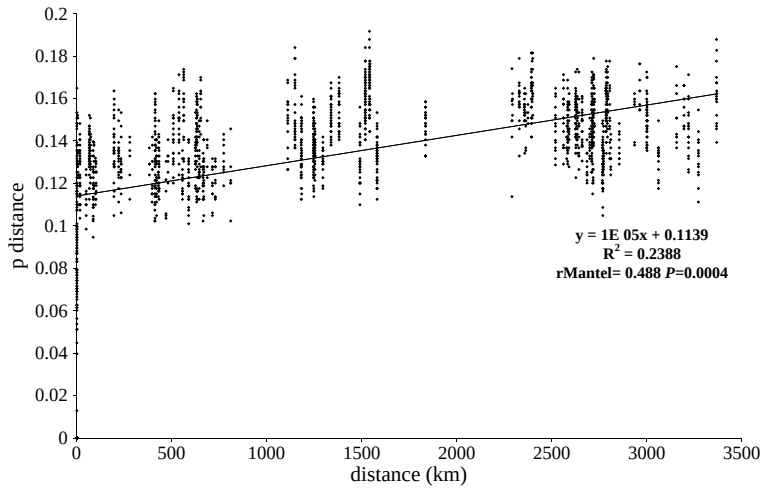

Fig. 3. Simple number of differences (p distance, using concatenated mtDNA COI + RAPD data) vs corresponding geographic distances among pairs of individuals of *D. pseudotsugae*.

Few population genetic studies of curculionids (including Scolytinae) have included RAPD markers (Cognato et al. 1995). However, mean expected heterozygosity (H_E) of *D. pseudotsugae* was 0.323 ± 0.001, greater than either *Diaprepes abbreviatus* L. (Bas et al. 2000) or *Tomicus piniperda* L. (Carter et al. 1996). RAPD markers have not been studied in other *Dendroctonus* species, but studies involving isozymes have demonstrated that they have a wide intra- and inter-population genetic variation. These studies have found higher observed heterozygosity than expected for random mating both in geographic populations (Stock et al. 1979, 1984; Six et al. 1999; Zúñiga et al. 2006), and in populations using different host trees (Stock and Amman 1980; Sturgeon and Mitton 1986; Langor and Spence 1991). High values of Nei and Shannon indices, as well as heterozygosity found in *D. pseudotsugae*, confirm these observations. In addition, genetic differences among populations are correlated geographically, as has been suggested for this and other *Dendroctonus* species using isozymes (Stock et al. 1979; 1984; Six et al. 1999; Zúñiga et al. 2006). The similar magnitude of genetic diversity of these indices suggests that genetic differences among populations are not an artifact associated with the sample size analyzed. While Nei's indices are affected by sample sizes of individuals as well as the number of loci analyzed (Nei 1978), the Shannon index is dependent on loci used but independent of the number of individuals analyzed (Hartl et al. 1994).

Comparison of bark beetle mtDNA nucleotide and haplotype diversity can be useful to assess demographic history of populations. High haplotype and low nucleotide diversity found in this study for *D. pseudotsugae* populations suggest that there has been rapid population growth from small populations, assuming sufficient time for recovery of haplotype variation via mutation but too short for the accumulation of large sequence differences (Grant and Bowen 1998). The same relationship between haplotype and nucleotide diversity has been observed in other Scolytinae, including *Dendroctonus* species (Cognato et al. 2005a; Maroja et al. 2007; Mock et al. 2007; Anducho-Reyes et al. 2008).

Independent factors such as length of the sequence, number of populations and individuals analyzed, as well as coalescence time, can bias estimations of genetic diversity. However, general patterns of variation in diversity indices were homogeneous among populations. These results indicate homogenization of interpopulation genetic diversity by high gene flow through extensive dispersal. Nevertheless, the dissimilar haplotype richness (number of haplotypes per population) observed among populations and population groups suggest that populations have experienced a recent reduction of their effective population size. Several studies carried out with European scolytines suggest that reduction and rapid population expansion might be the result of glacial or postglacial events that occurred during the Plio-Pleistocene period in Europe, which promoted contraction and expansion of beetle habitats (hosts) and postglacial migration processes from Plio-Pleistocene refugia (Stauffer et al. 1999; Horn et al. 2006). AMOVA and SAMOVA analyses indicate a strong genetic differentiation between Canada–USA and Mexico populations of this bark beetle, probably as a consequence of fragmentation of distribution range of its host plant (*P. menziesii* var. *glauca*) during climatic oscillations of the Plio-Pleistocene period. To infer the effect of glacial and postglacial Pleistocene events on genetic structure of

this species, it would be necessary to carry out a phylogeographic analysis including more populations given that historical inferences are biased by the number of population sampled (Petit et al. 2005).

Genetic structure

Several studies have suggested that specialization in host use may be a very important factor influencing genetic structure and differentiation of phytophagous insects (Van Zandt and Mopper 1998). The expectation of this hypothesis is that insect species with restricted diet breadth (specialist species) should be more prone to genetic differentiation than a generalist species because the distribution of the specialist's single host is patchier and less dense than the combined distribution of all generalist's hosts (Thompson 2004). This hypothesis has been tested in generalist and specialist species of *Dendroctonus* using different molecular markers (Kelley et al. 1999, 2000; Zúñiga et al. 2006). In these studies, generalist species showed slight genetic differences among populations colonizing different hosts in the same locality and cumulative differences among geographically isolated populations. Our results seem to support these expectations, because they reveal a strong pattern of differentiation between geographically separated populations, although it is difficult to know whether this differentiation was solely promoted by host fragmentation (due to both habitat loss and the breaking apart of habitat) or geographic distance.

Early genetic studies of *D. pseudotsugae* using isozymes suggest that differences between populations from Washington and Idaho ($S = 0.63$) were enough to consider them as two allopatric populations in the process of speciation (Stock et al. 1979; Bentz and Stock 1986). This conclusion was reinforced because the source populations utilized different varieties of *P. menziesii*. However, other studies have shown even higher similarity coefficients for different species (e.g. $S = 0.83$ in *D. jeffreyi* Hopkins and *D. ponderosae*) (Higby and Stock 1982). Our results did not reveal sufficient genetic differentiation among northern populations, which includes at least one population found on *P. m.* var. *menziesii*, to support the notion of ongoing speciation. The genetic differentiation that we found between *D. pseudotsugae* populations from north and south supports the expectation that geographical isolation, and probably habitat fragmentation, are primary factors affecting genetic structure. Although host use remains to be tested directly, the limited genetic differentiation among northern *D. pseudotsugae* populations suggests that host use has little effect on the genetic structure of this species. In addition, the absence of an observable pattern of isolation by distance within each north and south population group of *D. pseudotsugae* indicate that they have not yet reached gene flow-drift equilibrium, which suggest genetic drift is less influential than gene flow, no matter how long the separation among populations (model III, Hutchinson and Templeton 1999). These data agree with those obtained in other studies using various markers (isozyme, RAPD, mitochondrial) in *Conophthorus* Hopkins (Cognato et al. 2005b), *D. brevicomis* (Kelley et al. 1999), *D. jeffreyi* (Six et al. 1999), *D. ponderosae* (Kelley et al. 2000), *D. mexicanus* (Zúñiga et al. 2006), *Ips typographus* L. (Stauffer et al. 1999), and *Tomicus piniperda* L. (Carter et al. 1996). Our observations also suggest that the genetic structure of specialist species is more sensitive to IBD than generalist species, possibly because genetic differentiation of generalists is minimized due to different host use throughout its geographical distribution.

Isolation by distance

Linear regressions of both F_{ST} and p-distance vs geographic distance have shown a significant increase in amount of genetic differentiation among the populations we sampled, relative to the increase of geographic distance. These results agree with the study of Peterson and Denno (1998), which showed that the balance between dispersal and geographic isolation has had a greater effect over genetic structure and differentiation of phytophagous populations than specialization in the host-use. The genetic differentiation among pairs of populations estimated by $F_{ST}/1–F_{ST}$ (using both markers) also suggests that the break in the distribution of genetic variation, leading to isolation between North American and Mexican populations of *D. pseudotsugae*, is a result of limited gene flow and the consequent increase in genetic drift (Hutchinson and Templeton 1999).

Unfortunately, the methods used do not allow us to test what historical processes or factors have affected the genetic structure of *D. pseudotsugae*, because classical models of population genetics were designed to be independent of history (e.g. equilibrium models) and of the geographical landscape (e.g. non-structured island models). In addition, as some population genetic models in which a geographical perspective has been introduced, such as isolation by distance or stepping-stone, the landscape is considered uniform, isotropic, and not linked to the history of the population (Epperson 2003). However, low observed genetic differences within distinct geographical populations of the Douglas-fir beetle, and the finding that a model of isolation-by-distance is described by all populations that we analyzed, both support the

general premise that geographical isolation has played a fundamental role in the differentiation of its populations.

Finally, our results confirm that *D. pseudotsugae* populations from North America and those from Mexico on *P. menziesii* var. *glauca* are two true subspecies: *D. p. pseudotsugae* Hopkins and *D. p. barragani*, as was reported by Furniss (2001). *D. p. barragani* was collected from a single population in Chihuahua, Mexico, and described based on morphological characters, gallery differences, and intra- and inter-population mating tests. At the time, this was the only known population of *D. pseudotsugae* in Mexico, where its host *P. menziesii* is a relict species occurring in isolated locations in Chihuahua, Durango and Coahuila.

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APPENDIX 1

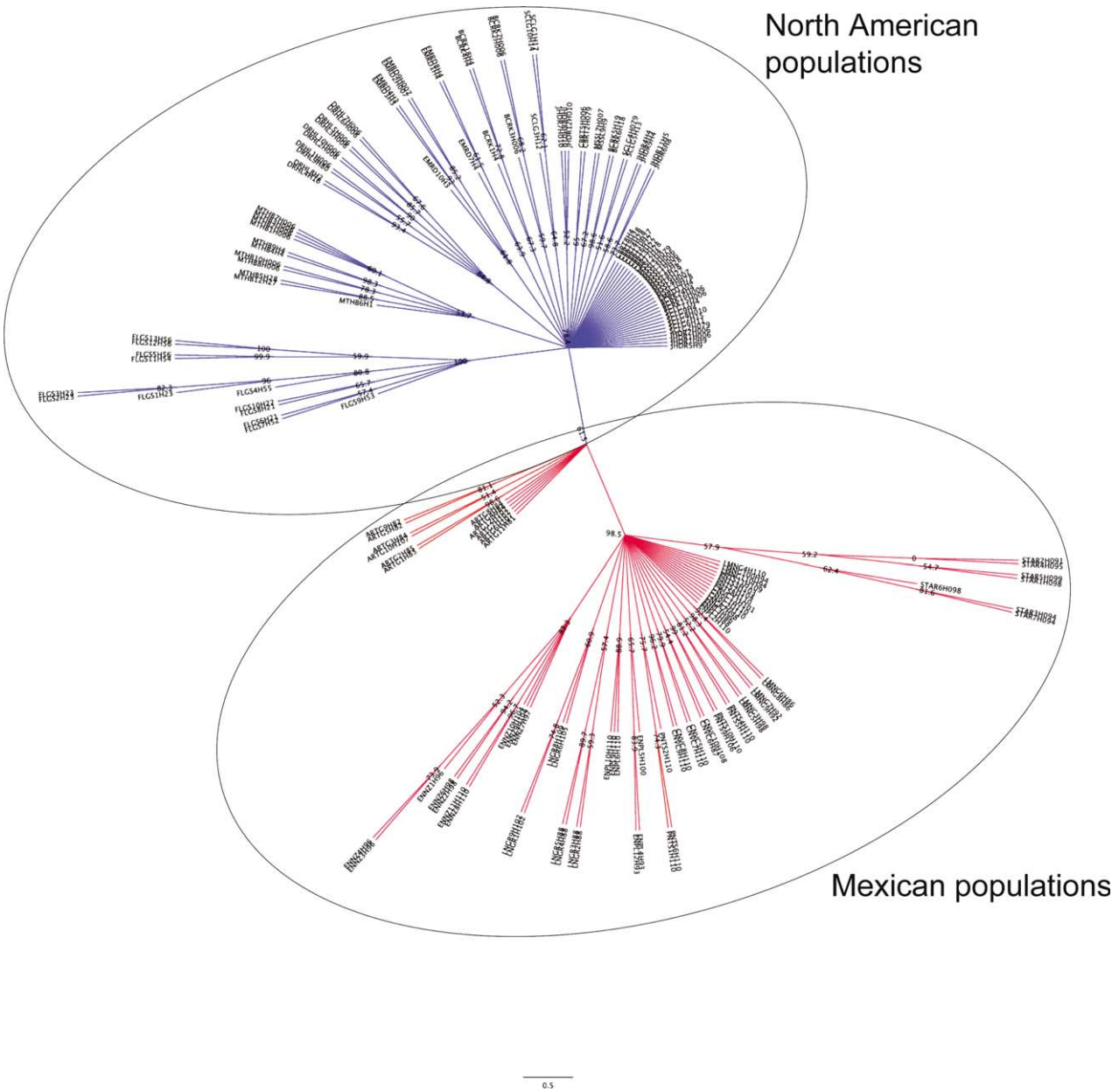

Fig. A1. Dendrogram constructed by neighbour-joining (NJ) with the p distance using PAUP*. The p distance allowed performing a simple comparison of number of differences among individuals considering both data sets (mitochondrial and RAPD data from each individual were concatenated). Bootstrap values at nodes (1000 pseudoreplicates)